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Site of Attachment of 11-*cis*-Retinal in Bovine Rhodopsin†

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ABSTRACT: A dipeptide containing the binding site for retinal in bovine rhodopsin has been isolated and its sequence determined. Rhodopsin containing [11-^{3}H]retinal was prepared in chromatographically pure form, and the [^{3}H]retinal was reductively linked to its binding site on opsin by using borane–dimethylamine. The [^{3}H]retinylopsin in octyl glucoside was exhaustively digested with Pronase, and its peptides were separated on silica gel in chloroform/methanol/ammonia [Bownds, D. (1967) *Nature (London) 216*, 1178–1181] followed by silica gel thin-layer chromatography in two solvent systems. The major retinyl peptide was shown to be alanyl-N^{ϵ}-retinyllysine by amino acid composition, ^{3}H content, and amino acid sequence analysis. The retinyl binding site is located in the carboxyl-terminal region of rhodopsin: when rod cell disk membranes containing [^{3}H]retinal rhodopsin were digested with thermolysin and then reacted with sodium borohydride or borane–dimethylamine, [^{3}H]retinal was reduced onto the F2 ($M_r \simeq 6000$) fragment, which derives from rhodopsin's carboxyl-terminal region.

Rhodopsin is the photoreceptor protein of rod cells in the vertebrate retina. Rhodopsin consists of an approximately 38 000-dalton protein, opsin, to which is bound a molecule of 11-*cis*-retinal. During the visual cycle, interaction of light with rhodopsin causes isomerization of the 11-*cis*-retinal to *all-trans*-retinal and its eventual dissociation from its binding site on the protein (Wald, 1968). For completion of the visual cycle and regeneration of the photosensitive visual pigment rhodopsin, a series of enzymes in the rod cell and pigment epithelium act to reconvert *all-trans*-retinal to 11-*cis*-retinal, which then recombines with the apoprotein opsin to regenerate rhodopsin (Bridges, 1976). The nature of the mode of association of retinal with the protein opsin has long been of interest due to the observation that retinal undergoes a red shift from its λ_{max} at 380 nm to as great as 560 nm in the formation of some visual pigments [see Hubbard et al. (1971)]. This shift is due both to the hydrophobic environment in the protein binding pocket and to the nature of the covalent linkage of retinal to the protein. A number of chemical studies have demonstrated that retinal is bound to the ϵ-amino group of a lysine residue in opsin via an aldimine or Schiff-base linkage [Bownds, 1967; Akhtar et al., 1968; reviewed by Knowles & Dartnall (1977)]. Spectroscopic studies reveal that the Schiff base is protonated [Lewis et al., 1973; reviewed by Callendar & Honig (1977)]. After the light-induced isomerization of protein-bound 11-*cis*-retinal to *all-trans*-retinal, the Schiff-base linkage becomes exposed to solvent and becomes susceptible to reaction with water or other reagents. It is then possible experimentally to covalently attach retinal to its protein-binding site by use of a variety of reducing agents which convert the Schiff-base linkage to a secondary amine (Bownds & Wald, 1965; Akhtar et al., 1965; Bownds, 1967; Hirtenstein & Akhtar, 1970; Zorn, 1971; Fager et al., 1972; Hall & Bok, 1976). In a pioneering study, Bownds (1967) enzymatically digested retinylopsin, isolated a set of overlapping peptides containing the retinyl binding site, and determined their compositions. There have been no subsequent reports elucidating the amino acid composition or primary structure of this region of the rhodopsin molecule. It has been the purpose of our study to isolate and characterize a small retinyl peptide from rhodopsin in order to assist in identifying the location of the retinyllysine in the primary structure of the protein.

Experimental Procedures

Materials

The following materials were obtained from Sigma Chemical Co.: *all-trans*-retinal, 9-*cis*-retinal, 13-*cis*-retinal, and N^{α}-acetyllysine. [11-^{3}H]Retinoic acid and 11-*cis*-retinal were gifts from Hoffmann-La Roche. Thermolysin, Pronase, and octyl β-D-glucopyranoside (octyl glucoside) were purchased from Calbiochem–Behring. Hydroxylapatite (DNA grade Bio-Gel HTP) and protein standard mixtures for molecular weight calibration of $NaDodSO_4$[1]–polyacrylamide gels were purchased from Bio-Rad. Silica gel HR was obtained from EM Laboratories, Inc. (Elmsford, NY), and silica gel G plates (250 μm) were obtained from Analtech, Inc. Diazomethane was synthesized from diazald (Fieser & Fieser, 1967). Diazald and borane–dimethylamine were purchased from Aldrich Chemical Co. Active manganese dioxide was prepared by the

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[1] Abbreviations used: DDT, dithiothreitol; [^{3}H]retinal ROS, ROS which have been bleached and then regenerated with [^{3}H]retinal; [^{3}H]-retinal rhodopsin, rhodopsin which has been prepared from [^{3}H]retinal ROS; $NaDodSO_4$, sodium dodecyl sulfate; Na_2EDTA, disodium ethylenediaminetetraacetate; TrTAB, tridecyltrimethylammonium bromide; Tris, tris(hydroxymethyl)aminomethane; ROS, rod cell outer segments; solvent I, $CHCl_3/CH_3OH/NH_4OH$ (70:27:3); solvent II, $CHCl_3/CH_3OH/NH_4OH$ (40:40:20); solvent III, 1-propanol/NH_4OH (70:30).

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method of Attenburrow et al. (1952). Lithium aluminum hydride was obtained from Alfa products. Dansyl chloride and fluorescamine were purchased from Pierce Chemical Co. All organic solvents used for the preparation of 11-*cis*-retinal by high-performance liquid chromatography were obtained from Burdick and Jackson Laboratories. All other chemicals were analytical reagent grade.

Methods

General Methods. Eyes were collected at a local slaughter house and kept on ice in the dark prior to dissection. Retinas were removed under dim red light and frozen quickly by dropping into liquid nitrogen. Retinas were stored in tightly wrapped containers at −70 °C.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. (1971), and urea–$NaDodSO_4$ polyacrylamide gel electrophoresis was performed according to Swank & Munkres (1971). Protein molecular weights were determined from a plot of log M_r vs. mobility with standard proteins for calibration. Molecular weight markers for the urea–$NaDodSO_4$ gels also included the cyanogen bromide peptides from cytochrome *c* and myoglobin. Gels for radioactive counting were sliced by using a Mickel gel slicer (Brinkman Instruments). Gel slices were solubilized by warming with 30% H_2O_2 (0.5 mL/1 mm of gel). Radioactive samples were mixed with a xylene–Triton X-114 cocktail (Anderson & McClure, 1973) containing Scinti-Prep (Fisher). Radioactive counting was performed with a Beckman LS7000 liquid-scintillation counter. Peptides were acid hydrolyzed in evacuated nitrogen-flushed vials with constant-boiling HCl at 107 °C for 21 h. Amino acid analysis was performed on a Beckman 119CL amino acid analyzer equipped with a Model 126 data system.

Preparation of 11-cis-[^{3}H]Retinal. [^{3}H]Retinoic acid (1.7 mg) was first reacted with diazomethane to form methyl retinoate. This ester was reduced with lithium aluminum hydride to [^{3}H]retinol by the method of Schwarzkopf et al. (1949), as modified by D. Bok (personal communication). The concentration of retinol was determined spectrophotometrically by using $E^{1\%}_{325nm}$ = 1845 (Hubbard et al., 1971).

[^{3}H]Retinol was oxidized to [^{3}H]retinal by being stirred under nitrogen with a suspension of active manganese dioxide in petroleum ether for 90 min. *all-trans*-Retinal content was measured spectrophotometrically by using $E^{1\%}_{383nm}$ = 1510 (Hubbard et al., 1971).

The photoisomerization of *all-trans*-[^{3}H]retinal was performed in ethanol solution (~5 mg/mL) at a distance of 2 ft from a fluorescent light for 1–2 h. The separation of retinal isomers was carried out under dim red light with an Altex Lichrosorb Si 60 column (250 mm long × 10 mm i.d., 10-μm packing) connected to a Waters Model 6000 pump and a U6K injector. The solvent used for elution was 7% diethyl ether in hexane (A. Kropf, personal communication). The column effluent was continuously monitored at 340 nm with a Waters Model 440 detector. The position of elution of 11-*cis*-retinal and other retinal isomers was identified by comparison with authentic standards and is in agreement with an earlier report (Rotmans & Kropf, 1975). The eluant containing 11-*cis*-retinal was collected, dried under nitrogen, and redissolved in ethanol. It was used immediately for regeneration of bleached ROS. The quantity of 11-*cis*-retinal prepared was determined spectrally by using $E^{1\%}_{379nm}$ = 878 (Hubbard et al., 1971). The specific radioactivity of the 11-*cis*-[^{3}H]retinal was determined to be approximately 600 mCi/mmol.

Preparation of [^{3}H]Retinal Rhodopsin. Rod outer segments (ROS) were prepared by using the method of Papermaster & Dreyer (1974), as modified by McDowell & Kühn (1977). The ROS were then washed three times in 67 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ and 1 mM DTT, resuspended at a rhodopsin concentration of approximately 3 mg/mL, and bleached at room temperature under a flood light for 90 min. 11-*cis*-[^{3}H]Retinal was mixed with a measured amount of nonradioactive 11-*cis*-retinal, and a fivefold molar excess was added to the freshly bleached ROS. The suspension was mixed thoroughly and incubated in the dark at room temperature for 3–4 h. Bleached ROS were regenerated to the extent of 85–95% by this procedure. The regenerated ROS ([^{3}H]retinal ROS) were washed with 1% hydroxylamine hydrochloride in 67 mM potassium phosphate buffer (pH 7.0), pelleted, and washed three times with cold deionized water. Rhodopsin containing 11-*cis*-[^{3}H]retinal ([^{3}H]retinal rhodopsin) was purified by chromatography on hydroxylapatite with the detergent TrTAB (Hong & Hubbell, 1972).

Reduction of [^{3}H]Retinal Rhodopsin in ROS Membranes. Two procedures, employing different reducing agents, were compared. When sodium borohydride was used as the reducing agent, [^{3}H]retinal ROS were suspended in 67 mM sodium phosphate buffer (pH 7.0) at a rhodopsin concentration of 2 mg/mL and the pH was raised to 8.0 with 1 N NaOH (Bownds, 1967). A 1000× molar excess of sodium borohydride was added, and the suspension was immediately irradiated at room temperature with a 100-W white light for 5 min. An equal volume of $NaDodSO_4$ dissociation medium was added and the sample examined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

[^{3}H]Retinal ROS were also reduced by using borane–dimethylamine (Hall & Bok, 1976). Samples were prepared for electrophoretic analysis as described above.

Reduction of Purified [^{3}H]Retinal Rhodopsin in Solution. Purified [^{3}H]retinal rhodopsin, prepared by chromatography on hydroxylapatite by using TrTAB, was dialyzed to remove detergent and collected as a pellet by centrifugation. The pellet was dissolved in 67 mM sodium phosphate (pH 7.0) containing 50 mM octyl glucoside. The solution was diluted to yield a concentration of 2 mg/mL in rhodopsin and 30 mM in octyl glucoside. An aliquot was reduced by using sodium borohydride as described for reduction of [^{3}H]retinal ROS. A separate aliquot was reduced by using borane–dimethylamine on the basis of the procedure of Hall & Bok (1976). Borane–dimethylamine was added to yield a ratio of 20 mg/mg of rhodopsin, followed immediately by addition of 6 N HCl to make the reaction mixture 0.1 N (pH ~1.2 to 1.6). Incubation was performed at room temperature for 10 min followed by neutralization with 6 N NaOH. Samples were diluted with equal volumes of $NaDodSO_4$ dissociation medium prior to electrophoresis.

Thermolysin Digestion of [^{3}H]Retinal ROS. Thermolysin digestion of [^{3}H]retinal ROS was performed according to Saari (1974) except that a higher concentration of thermolysin (7% by weight of rhodopsin) was used. Digestion was terminated by addition of Na_2EDTA to a final concentration of 10 mM. ROS were washed twice with deionized water and then suspended in 67 mM sodium phosphate buffer (pH 7.0) prior to reduction. Reduction was carried out as described previously by using sodium borohydride. The thermolysin-digested and $NaBH_4$-reduced ROS were washed three times with deionized water and dissolved in $NaDodSO_4$–urea dissociation medium to a protein concentration of 1 mg/mL (Swank & Munkres, 1971). Aliquots were taken for $NaDodSO_4$–urea polyacrylamide gel electrophoresis.

Pronase Digestion of [^{3}H]Retinylopsin. Purified [^{3}H]retinal rhodopsin, in 30 mM octyl glucoside, was reduced with borane–dimethylamine as described previously. It was dialyzed against deionized water containing 1 mM DTT at 4 °C. The precipitated [^{3}H]retinylopsin was harvested by centrifugation at 40000*g* for 30 min. Tris–acetate (10 mM, pH 7.5), containing 50 mM octyl glucoside, was added to the pellet, yielding a turbid solution with a protein concentration of 3 mg/mL. Pronase digestion was carried out under nitrogen at 30 °C in the presence of sodium azide (Bownds, 1967). Fresh Pronase equal to 2% of the weight of [^{3}H]retinylopsin was added five times over a period of 48 h and the pH was maintained at 7.5 by occasional additions of 0.1 N NaOH. After 48-h incubation, the Pronase digest was centrifuged and the supernatant removed and lyophilized. Ethanol (100%) was added to the lyophilized supernatant to extract the [^{3}H]retinyl peptides. The extract was rotary evaporated and redissolved in 5–10 mL of $CHCl_3/CH_3OH/NH_4OH$ (70:27:3) (solvent I).

Silica Gel Column Chromatography of the Soluble Pronase Peptides of [^{3}H]Retinylopsin. Material from the Pronase digest of [^{3}H]retinylopsin which was soluble in solvent I was applied to a column of silica gel HR (2.5 × 25 cm) equilibrated in that solvent. Chromatography was carried out under 5-psi N_2 pressure (Bownds, 1967), and 2.2-mL fractions were collected by drop counting.

Purification of Peptides from Silica Gel HR Column by Thin-Layer Chromatography. The radioactive pool containing the greatest amount of [^{3}H]retinyl peptide from the silica gel HR column was dried under nitrogen, redissolved in 0.5 mL of solvent I, and spotted as a streak along the origin line of a silica gel G chromatography plate. Spotting was performed in a glove bag in a nitrogen atmosphere, under dim light. The plate was developed in the dark in a tank equilibrated with $CHCl_3/CH_3OH/NH_4OH$ (40:40:20) (solvent II). The retinyl peptide was visualized by UV illumination and scraped from the plate (band 1). The plate was then sprayed with fluorescamine, and peptide-containing regions were observed with UV illumination (bands 2 and 3). These bands were scraped from the plate, and all peptide material was eluted by extracting three times with 5 mL of solvent II. The eluted bands were dried under N_2, dissolved in solvent I, reapplied to a silica gel G chromatography plate, and developed with solvent II or solvent III. Peptide-containing bands were identified and eluted as described above. Aliquots (7.5%) of each eluted band were taken for acid hydrolysis and amino acid analysis.

Preparation of N^{α}-Acetyl-N^{ϵ}-retinyllysine. Commercial N^{α}-acetyllysine (Sigma) was further purified by thin-layer chromatography; 16.6 mg was dissolved in 60% CH_3OH and spotted on a silica gel G thin-layer plate which was then developed in solvent I. Two contaminants had mobility in this solvent, whereas the N^{α}-acetyllysine remained at the origin from which it was eluted with 60% CH_3OH.

N^{α}-Acetyl-N^{ϵ}-retinyllysine was prepared as previously described (Bownds, 1967). It was further purified by thin-layer chromatography on silica gel G in solvent I. Its concentration was measured spectrophotometrically by using $E_{333nm} = 50000$ (Bownds, 1967).

Results

Reduction of [^{3}H]Retinal Rhodopsin in ROS. The Schiff-base linkage which binds retinal to opsin may be successfully reduced by either sodium borohydride or borane–dimethylamine to form [^{3}H]retinylopsin. The results of the borane–dimethylamine reduction are shown in Figure 1. The same results were obtained when sodium borohydride was used. The peak of radioactivity found at $R_f = 0.56$ corresponds to $M_r = 35000$ which is the apparent molecular weight found for opsin with this gel system (Papermaster & Dreyer, 1974). When the reduced ROS are analyzed with the $NaDodSO_4$–urea gel system of Swank & Munkres (1971), some opsin dimer is formed although the majority of the protein-bound ^{3}H migrates with the opsin monomer (Figure 2A). The fast-migrating ^{3}H-containing component which is present in Figure 1 (92–104 mm) is seen more clearly in Figure 2 (55–70 mm, $R_f \simeq 0.67$). This ^{3}H-containing material fails to stain with Coomassie blue and migrates in this gel system with authentic retinylphosphatidylethanolamine, whereas retinal, retinol, and retinyl oxime are clearly separated, with $R_f \simeq 0.90$. Since excess [^{3}H]retinal was added to ROS in the regeneration of opsin, it is not unexpected that [^{3}H]retinylphosphatidylethanolamine should be formed.

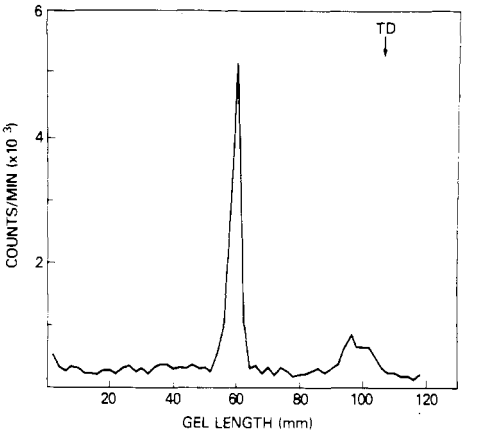

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of [^{3}H]-retinal ROS reduced with borane–dimethylamine. [^{3}H]Retinal ROS were reduced with borane–dimethylamine as described under Experimental Procedures. A sample containing 44 μg of retinylopsin was submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis in 5.6% polyacrylamide gels by the procedure of Fairbanks et al. (1971). The gel was fixed and sliced into 2-mm sections for determination of radioactivity. The major peak of radioactivity (54–62 mm) corresponds to the position of opsin in a duplicate gel stained with Coomassie blue. Gel top is at left of figure; TD = tracking dye.

Thermolysin Fragment F2 Contains Rhodopsin's Retinyl Attachment Site. [^{3}H]Retinal ROS were digested with thermolysin in order to produce the noncovalently associated membrane-bound rhodopsin fragments F1 and F2. Sodium borohydride reduction was performed in order to covalently link the [^{3}H]retinal to its protein-binding site. The ROS were then analyzed by $NaDodSO_4$–urea polyacrylamide gel electrophoresis (Figure 2B). Radioactivity is clearly seen to be associated with fragment F2, the smaller of the two membrane-bound proteolysis products of rhodopsin. The F2 fragment has $M_r \simeq 6000$ in this gel system.

Purification and Reduction of [^{3}H]Retinal Rhodopsin. [^{3}H]Retinal rhodopsin was obtained in pure form by hydroxylapatite chromatography of ROS which had been solubilized in the detergent TrTAB. Rhodopsin prepared by this method had $A_{280}/A_{498} = 1.7$ and a specific radioactivity of 8.7×10^4 dpm/nmol. TrTAB was removed by dialysis, and a rhodopsin solution was then prepared in octyl glucoside. Reduction was performed with either sodium borohydride or borane–dimethylamine. Analysis of the products from both reductions by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that approximately 80% of the radioactivity had been linked to rhodopsin and that most of the rhodopsin was present as high-molecular-weight aggregates. However, when the

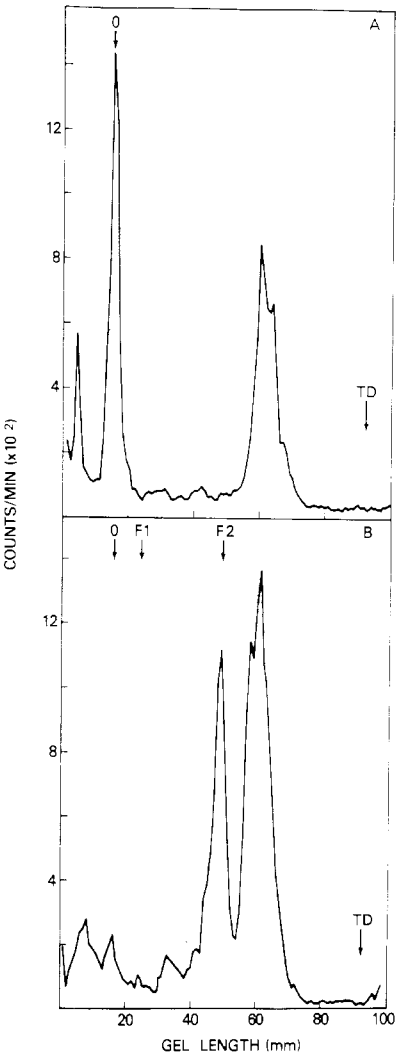

FIGURE 2: (A) $NaDodSO_4$–urea polyacrylamide gel electrophoresis of [^{3}H]retinal ROS reduced with sodium borohydride. [^{3}H]Retinal ROS were reduced with sodium borohydride as described under Experimental Procedures. A sample containing 30 μg of retinylopsin was submitted to $NaDodSO_4$–urea–polyacrylamide gel electrophoresis in 12.5% polyacrylamide gels by the procedure of Swank & Munkres (1971). The gel was fixed and sliced into 1-mm sections for determination of radioactivity. Position of migration for opsin (0) was determined from a duplicate gel stained with Coomassie blue. TD = tracking dye. (B) $NaDodSO_4$–urea–polyacrylamide gel electrophoresis of thermolysin-digested [^{3}H]retinal ROS reduced with sodium borohydride. [^{3}H]Retinal ROS were digested with thermolysin and reduced with sodium borohydride as described under Experimental Procedures. A sample containing 35 μg of retinylopsin was submitted to $NaDodSO_4$–urea–polyacrylamide gel electrophoresis. The gel was fixed and sliced into 1-mm sections for determination of radioactivity. Positions of migration for opsin (0), F1, and F2 were determined from a duplicate gel stained with Coomassie blue. TD = tracking dye. Absolute amounts of radioactivity in the gels from (A) and (B) cannot be directly compared since the samples are from different experiments.

strong reducing agent sodium borohydride was used, some ^{3}H was also found at $M_r \simeq 18\,000$, presumably due to peptide bond cleavage of rhodopsin. Our further studies were performed with the milder reducing agent borane–dimethylamine.

Pronase Peptides of [^{3}H]Retinylopsin. [^{3}H]Retinal rhodopsin was mixed with nonradioactive rhodopsin to give 3.8 μmol of rhodopsin of specific radioactivity 8.4×10^3 dpm/nmol. This was dissolved in octyl glucoside and reduced by using borane–dimethylamine. The [^{3}H]retinylopsin produced was exhaustively digested with Pronase. Peptides solubilized by the enzymatic digestion were extracted as described under Experimental Procedures and submitted to silica gel column

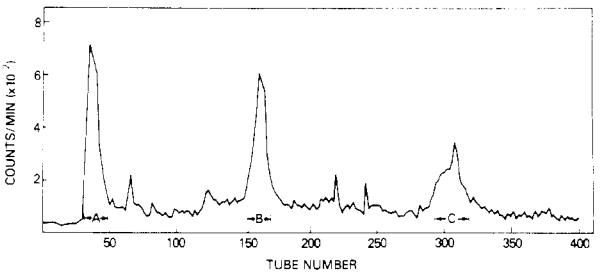

FIGURE 3: Pronase peptides of [^{3}H]retinylopsin. Peptides from a Pronase digest of 3.8 μmol of [^{3}H]retinylopsin (specific radioactivity 8.4×10^3 dpm/nmol) were applied to a 2.5 × 25 cm column of silica gel HR, as described under Experimental Procedures. The column was eluted with $CHCl_3/CH_3OH/NH_4OH$ (70:27:3) and 2.2-mL fractions were collected. Samples of 50 μL were taken for radioactivity determination.

Table I: Purification of a [^{3}H]Retinyl Peptide from [^{3}H]Retinal Rhodopsin

step	sample	percent
1	[^{3}H]retinal rhodopsin	100
2	[^{3}H]retinylopsin	~80[a]
3	supernatant following Pronase digestion	30.3[b]
4	ethanol-soluble material	24.4
5	peak B, silica gel column	3.1
6	first preparative TLC, band 1	2.4
7	second preparative TLC, band 1a	2.0

[a] On the basis of gel electrophoresis data. [b] An equivalent amount of retinyl compounds similar to those in the supernatant were found in the "insoluble" material in a comparable experiment.

chromatography (Figure 3). Three major peaks of radioactivity (designated A, B, and C in Figure 3) were found in the column eluate. Fractions from the column eluate were further evaluated by thin-layer chromatography (Figure 4). Fractions comprising radioactive peak A (Figure 3) were found to contain essentially no peptides but to possess fluorescent material which cochromatographs with retinol (by thin-layer chromatography). Fluorescamine-reactive peptides were found in all other column fractions examined. Radioactive peak B contained 12.8% of the total radioactivity applied to the column in the experiment reported in Table I (in a separate experiment this value was 18.2%). Thin-layer chromatographic analysis of fractions from peak B demonstrated the presence of an intrinsically fluorescent peptide (i.e., a retinyl peptide) as well as several additional peptide-containing bands (Figure 4). Peak C appeared to contain smaller amounts of a retinyl peptide of mobility less than that of the retinyl peptide in peak B, along with larger amounts of nonretinyl peptides. Peak C was not investigated further.

Peak B (Figure 3) was pooled and submitted to preparative thin-layer chromatography in solvent II. Three major bands were resolved, one of which (band 1) was intrinsically fluorescent and contained the bulk of the radioactivity. Each band was eluted separately and rechromatographed as described under Experimental Procedures. This resulted in the further resolution of each band into two components. The amino acid analysis, percent recovery, and amino acid sequence results for each of the six bands are presented in Table II. Band 1a is the only one which contains a retinyl peptide. The retinyl peptide in band 1a is determined to be pure by several criteria: it migrates as a homogeneous component by thin-layer chromatography in two solvent systems, has an amino acid analysis which yields a small whole number ratio of amino acids, and gives a single unique amino acid sequence. The retinyllysine is therefore found in an alanyllysine sequence. None of the

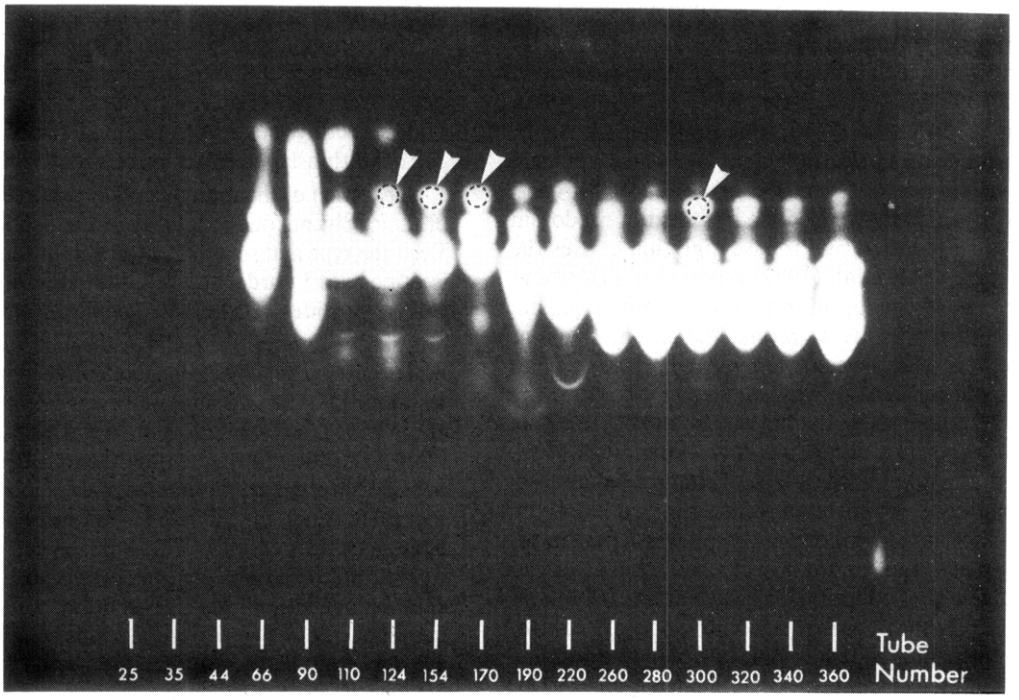

FIGURE 4: Thin-layer chromatogram of selected fractions from silica gel chromatography of a Pronase digest of [^{3}H]retinylopsin. Aliquots (500 μL) of selected fractions from silica gel column chromatography (Figure 3) were concentrated and spotted for silica gel G thin-layer chromatography. The plate was developed in the dark in $CHCl_3/CH_3OH/NH_4OH$ (40:40:20). Intrinsically fluorescent materials (retinoids and retinyl peptides) were visualized under a long-wave UV lamp and their positions are shown by arrows to dotted circles. The plate was then developed with fluorescamine in order to visualize peptides and photographed under UV illumination as shown above.

Table II: Analysis of Peptide-Containing Bands Obtained by Preparative Thin-Layer Chromatography[a]

	band					
amino acid	1a	1b	2a	2b	3a	3b
Thr		0.96	0.17		0.86	0.82
Pro		1.00	2.12	1.40		1.75
Gly				0.89		
Ala	*1.0*	1.33	*1.0*	0.16	*2.0*	*2.0*
Val			0.25			1.01
Ile		*1.0*	0.15	*1.0*	0.97	1.18
Leu			0.96	0.14		
Tyr			0.10			0.10
Phe				1.06		0.30
Lys	0.77[b,c]		0.19	0.10	0.37	0.11
% recovery[d]	2.1	0.40	0.75	0.95	0.17	1.5
R_f solvent II	0.75	0.56, 0.72	0.63, 0.68	0.63	0.63	0.63
R_f solvent III	0.59	0.42, 0.64, 0.76	0.57	0.60	0.41	0.52
N-terminal[e]	Ala	ND[f]	Ala (Val)	Gly	ND	Ala, Val, Thr
N-1	-	ND	Ile (Leu) (Val)	Pro	Ile	Ile, Ala, Phe, Val

[a] Amino acid analysis is corrected for "blank" values obtained by hydrolysis of an equivalent amount of silica gel. Molar values of amino acids are normalized to the residue whose value is italicized. Amino acids present in less than 0.10 mol/mol of "peptide" are not reported. [b] Value is corrected for destruction of retinyllysine. [c] 0.97 mol on the basis of retinal ^{3}H content. [d] Based on mol of amino acid used for normalization and initial amount of rhodopsin. [e] Residues in parentheses indicate detectable but faint. [f] ND = not determined.

other fluorescamine-staining components separated from pool B (Table II) contain retinal, and none is composed of a single pure peptide.

Discussion

We have obtained the first amino acid sequence information for rhodopsin which contains the retinyl attachment site. These results, in conjunction with more complete sequence information for rhodopsin, should allow identification of the site of retinal attachment in the protein's primary sequence. Our approach to the isolation of the retinyl peptide relied heavily on the pioneering work of Bownds (1967). We were able to take advantage of many developments in the intervening years in order to improve and extend work on the isolation, purification, and characterization of retinyl peptides.

To initiate these studies, we reduced [^{3}H]retinal rhodopsin to form [^{3}H]retinylopsin while rhodopsin occupied its native location in ROS membranes. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis showed that the protein-bound [^{3}H]retinal comigrated with the protein opsin, in agreement with previous reports describing the use of the reducing agents sodium borohydride (Papermaster & Dreyer, 1974) and borane–dimethylamine (Hall & Bok, 1976). In an attempt to localize a smaller defined region of the rhodopsin molecule which carries the retinyl attachment site, we have reduced the two-fragment rhodopsin complex formed by thermolysin digestion of ROS membranes. Reduction of this [^{3}H]retinal F1–F2 complex shows that the retinyl attachment site resides in the smaller F2 fragment. This fragment has been shown to originate from the carboxyl-terminal region of rhodopsin

(Hargrave & Fong, 1977) and has $M_r \simeq 6000$ when analyzed by $NaDodSO_4$–urea polyacrylamide gel electrophoresis. This is in agreement with some previous findings following digestion of rhodopsin in ROS with thermolysin (Pober & Stryer, 1975), papain (Fung & Hubbell, 1978), and subtilisin (J. Saari, personal communication; J. Gaw and E. Dratz, personal communication). In contrast, van Breugel et al. (1975) report that the large N-terminal glycopeptide fragment of rhodopsin (comparable to F1) formed by Pronase digestion carries the retinyl binding site. Sale et al. (1977) report that a third and different fragment of rhodopsin formed by extensive papain digestion of ROS is the site of retinal reduction. Papain-cleaved ROS were also investigated by Albert & Litman (1978) who found retinal is always reductively fixed to the small F2-like fragment as well as to one or the other of the remaining two fragments, depending upon experimental conditions. Due to the great variety of reducing agents and experimental conditions employed in the studies just cited, the reasons for lack of agreement cannot be readily determined. We chose to study the comparatively well-characterized thermolysin-digested rhodopsin (Hargrave et al., 1980) and to employ either sodium borohydride with good pH control or borane–dimethylamine at low pH, in order to minimize retinal migration by transiminization (Daemen et al., 1971). Our findings that both sets of reducing conditions lead to attachment of retinal to F2 and that one predominant peptide is found from the Pronase digest suggest that specificity has been achieved under our conditions of reduction. It should be possible to perform one further test to determine if any significant amount of migration of the retinal had occurred under the conditions used for reduction. If any retinal had not been reduced to its native binding site, addition of 11-*cis*-retinal in the dark to the reduced opsin would regenerate rhodopsin's characteristic absorption at 498 nm. Although we did not perform this experiment during our current study, data from such an experiment should also serve to further quantify the efficiency of the reduction step.

During the course of these studies we observed that sodium borohydride reduction of rhodopsin in detergent solution caused fragmentation of rhodopsin's polypeptide chain. Although there have been no previous reports that sodium borohydride degrades rhodopsin in detergent solution, it has previously been observed that sodium borohydride can cleave peptide bonds in proteins (Crestfield et al., 1963; Seon et al., 1965; Andersson, 1969).

For preparative studies on the peptide binding site of retinal, we chose to employ chromatographically purified [^{3}H]retinal rhodopsin. Use of the radioactive derivative allowed us to perform quantitative studies. Octyl glucoside was selected as the detergent since it is not only a very gentle detergent but also, in contrast to digitonin, it can produce concentrated solutions of rhodopsin and is easily removed (Stubbs et al., 1976). Our reduction of [^{3}H]retinal to opsin in octyl glucoside gave an approximately 80% yield, which compares well to reduction in digitonin solution under a variety of conditions (Hirtenstein & Akhtar, 1970). We digested [^{3}H]retinylopsin separately on an analytical scale with trypsin, chymotrypsin, thermolysin, and Pronase. Our results agree with those of Bownds (1967) that the largest amounts of soluble retinyl peptides are obtained by the use of Pronase (J. K. Wang and P. A. Hargrave, unpublished experiments). Since Pronase consists of a mixture of proteolytic enzymes of diverse specificities (Narahashi, 1970), we performed an exhaustive digestion in hopes of minimizing the number of different peptides produced and thereby maximizing their yield. This goal seems to have been achieved inasmuch as only one major (and one minor) retinyl peptide was initially detected by the combined use of column chromatography and thin-layer chromatography (Figures 3 and 4). However, small amounts of other retinyl peptides could be detected in peak C (Figure 3) if the thin-layer plates were greatly overloaded.

Separation of the retinyl peptide required the use of alkaline organic solvents due to lability of the peptide in acidic media. Even the type and source of silica gel for the column separation is important in order to obtain satisfactory resolution of the retinyl peptides (D. Bownds, personal communication).

In order to obtain the [^{3}H]retinyl peptide in pure form, it was necessary to subject the pool enriched in this peptide (pool B, Figure 3) to two successive purifications by thin-layer chromatography. More than five other peptides were separated from the retinyl peptide; the number of contaminating peptides present is not certain since the additional chromatographic bands which were resolved still did not contain pure homogeneous peptides (Table II). The [^{3}H]retinyl peptide was shown to be homogeneous by amino acid analysis, amino acid sequence analysis, ^{3}H content, and chromatographic homogeneity in two solvent systems. Such multiple criteria are important as proof of peptide purity. We find the retinyl binding site in an alanyllysine sequence. This is consistent with the retinyl peptide *compositions* reported by Bownds (1967), all of which contain alanine as well as lysine. Although it is possible that the multiple retinyl peptides described by Bownds (1967) do in fact represent unique larger peptides from the same site, their homogeneity would need to be more completely established by using the criteria listed above.

The nature of the *insoluble* peptide material following Pronase digestion was further investigated. In one experiment in which the supernatant contained 44% (and the pellet 56%) of the radioactivity, the insoluble pellet was subjected to ethanol extraction and an additional 31% of the initial radioactivity was solubilized. This results in a total of 75% of the radioactivity in the Pronase digest being soluble in ethanol. When the ethanol-soluble material from the pellet was examined by silica gel column chromatography, an elution profile was obtained which was nearly identical with that obtained for the supernatant. Of the radioactivity applied to the column, 27.9% was found in pool B, and the same retinyl peptide was prepared by thin-layer chromatography. Thus, the true yield of retinyl peptide is much greater than that shown in Table I. The distinction between "soluble" and "insoluble" peptide material appears to be an artificial one presumably based upon the relative ratios of peptide material and detergent in the digest. The entire digest could be prepared for column chromatography rather than just the supernatant. However, it would be important to increase the size of the column to accommodate the greater load of material. In experiments in which our load exceeded the capacity of the silica gel column, peak B became broader and less well resolved and yielded more complex peptide mixtures from which a pure retinyl peptide could not be obtained by repeated thin-layer chromatography.

In work with the retinyl protein and peptides it was important to minimize exposure to light and oxygen, to work rapidly, and to keep manipulations to a minimum. In an early experiment in which we did not observe these precautions and delayed performing the second thin-layer chromatography step until the following day, our yield of [^{3}H]retinyl peptide decreased from 90 to 34 nmol (Wang and Hargrave, unpublished experiments). This represents a 56% loss of material at this step compared with a 17% loss observed when appropriate

precautions were taken (Table I).

Due to the use of [^{3}H]retinal, we were able to conveniently measure the amount of retinyllysine by radioactivity. We found, however, that it was possible to regenerate some lysine from the retinyl peptide following acid hydrolysis. In order to measure the extent of lysine regeneration, we prepared N^{α}-acetyl-N^{ϵ}-retinyllysine. Under our conditions of acid hydrolysis, lysine was regenerated in approximately 23% yield. This figure was used to correct for lysine destruction resulting from acid hydrolysis of the retinyl peptide.

The sequence alanyllysine is unlikely to occur more than once in the sequence of F2; thus the retinyl attachment site should be readily identified when the sequence of F2 from bovine rhodopsin is completed. However, a larger retinyl peptide should be conveniently prepared from a different enzymatic digest of retinylopsin based upon the procedures outlined above. It will be of great interest in the future to compare such sequence data from different visual pigments and attempt to determine the structural requirements in this region of the protein.

Added in Proof

A peptide from the carboxyl-terminal region of rhodopsin has been reported to contain the sequence alanyllysine (Pellicone et al., 1980). The peptide sequence, taken together with that of Hargrave et al. (1980), would place this lysine as the 53rd amino acid from rhodopsin's carboxyl terminus, i.e., within the sequence of fragment F2. The amino acid sequence immediately adjacent to this lysine contains the amino acids reported to be in the compositions of retinyl peptides from rhodopsin (Bownds, 1967). These data, taken together, serve to uniquely identify the site of retinal attachment in the primary structure of rhodopsin.

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